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MICROORGANISMS CAPABLE OF DEGRADING REFRACTORY HYDROCARBONS IN OHIO WATERS

By
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University of Dayton
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INTRODUCTION

Degradation of hydrocarbons by microorganisms was first observed nearly 80 years ago (Miyoshi, 1895), but remained a subject primarily of academic interest until the 1930's and 1940's when a number of applied aspects led to effective contacts between petroleum engineers and microbiologists for the purpose of developing petroleum technology. In the last 20 years, increasing use of petroleum products has been accompanied by growing concern over the impact of petroleum and its products on the environment. At the level of the ecosystem, microbiologists have resorted largely to simplified laboratory systems. This approach has been fruitful, but it often has involved study of the easiest organism to work with, under simplified conditions and on single, purified substrates.

Over 100 species of microorganisms in some 60 genera can oxidize one or more hydrocarbons. The organisms include bacteria, yeasts, and filamentous fungi. Although the filamentous fungi comprise a significant fraction of the organisms, they have received relatively little attention in comparison to the bacteria (Collins, 1963; ZoBell, 1969; Atlas and Bartha, 1972a; and others) and yeasts (Ahearn, Meyers, and Standard, 1971). Moreover, a number of studies have dealt with total microbial activity without regard to the types of organisms involved.

In addition, efforts have been expended mainly on those fractions of oil which are least troublesome ecologically: alkanes and simple alkenes; while cycloparaffins, aromatic hydrocarbons, and asphaltic compounds and alkylated derivatives, which together comprise 40-85% of crude oils, have received much less attention. These fractions persist in fresh water, marsh and marine waters (Horn, Teal, and Backus, 1970; Blumer and Sass, 1972; Hites and Biemann, 1972) where they can aggregate as petroleum lumps or "tarballs". Tarballs can support growth of microbes and marine organisms (Horn, et al., 1970). They can sink and rise under conditions which are poorly understood (Davis, 1967; Hartung and Klingler, 1968; Nelson-Smith, 1970). The recalcitrant compounds include carcinogens (Ames, Sims, and Grover, 1972) and a variety of compounds which are toxic to benthic and other organisms (McCauley, 1966; Blumer and Sass, 1972; Brown, 1972; Takahashi and Kittredge, 1973). In marine organisms, recalcitrant compounds can enter the food web, accumulating in mussels (Lee, Sauerheber, and Benson, 1972), shellfish (Blumer, Souza and Sass, 1970), and surface-feeding fish (Horn, et al., 1970), although some fish have systems for efficient detoxification and removal of at least some toxic hydrocarbons (Lee, Sauerheber, and Dobbs, 1972).

Last, more attention has been devoted to marine, estuarine, and marshland systems than to fresh-water systems. Few studies have dealt with shallow, fresh-water systems of the type which are common in Ohio. In the ocean or in deep lakes more microbial activity takes place in the upper 75 to 100m of the water column than in deeper parts of the water column. Paerl (1973) reported that in the upper 75m of Lake Tahoe, fungi and bacteria played a role in aggregation of detrital particles and in their decomposition. Microbial activity decreased in deeper waters but was noted in bottom sediments. In shallow estuaries and in marine marshes (ZoBell and Prokop, 1966) and in shallow lakes (Strzelczyk, Donderski, and Lewosz, 1972) such as Lake Erie and most Ohio lakes and streams, the mud-water interface is the scene of greater microbial activity than the water column. Suspended particulates, both detritus (Leshniowsky, et al., 1970; Paerl, 1973) and particles of clay (Tsernoglou and Anthony, 1971) can enhance microbial activity, presumably by providing adsorptive reaction sites. Microbial activity in sediments and on particulates should have a greater impact on the ecosystem in shallow lakes and rivers than in deep waters, because bottom-sediments can be mixed throughout the entire water column more easily than in deep waters.

The present work had as its objectives:

1. To determine the prevalence of hydrocarbon-using bacteria, yeasts and filamentous fungi in hydrocarbon-contaminated and non-contaminated water in Ohio, and to determine the relative numbers of each of those groups of microorganisms.

2. To determine the effect of hydrocarbon pollution on total microbial populations: hydrocarbon-users as well as those which do not use hydrocarbons.

3. To assemble a collection of microorganisms which would be representative of hydrocarbon-users found in fresh waters.

4. To assess the potential of such organisms as indicator organisms for hydrocarbon pollution and their potential for participating in degradation of hydrocarbons, particularly those refractory compounds which accumulate at the bottom of lakes and streams.

MATERIALS AND METHODS

Sampling sites. Four bodies of water and sampling sites on them are shown in Figures 1 through 4. Acton Lake (Fig. 1) in Heuston Woods State Park is a man-made recreational lake of about 600 surface acres. It receives limited hydrocarbons since recreational boats are limited to 10-h.p. motors. The main course of water is from a stream at site 4, through site 1 continuing along the North side of the Lake and over the spillway. Site 2 is near the South shore and is not in the main water course. Site 3 is at the marina where boats with outboard motors are docked from Spring through Fall. The water level in Acton Lake is lowered about 4 ft from late Fall until early Spring. Holiday Lake (Fig. 2) near Willard, Ohio is a man-made recreational lake of about 225 surface acres which received a heavy influx of diesel oil through one inlet. The Lake may also receive chronic hydrocarbon pollution from a sewage disposal plant, a railroad yard and from recreational boats. The morphology of the Lake permitted sampling near where oil originally gained access, at several points along the main watercourse through the Lake, and at other points which may not have been affected as seriously by the spill. Site 5 is close to where the stream bearing oily wastes enters the Lake. On October 1, 1970, over 4,000 gallons of diesel oil entered the Lake through this stream. An oil slick covered much of the Lake from site 5 past site 7 which is in the main watercourse downstream. Lesser amounts of oil were evident on the surface near the dam at site 8. Little or no oil was observed near the spillway. As a result of wind action, a thick surface layer of oil was present on much of the North shoreline along this route and tarry residues accumulated on docks and on the shoreline. No evidence of that accumulation was noted when the present study began in July, 1972. But bottom sediments taken at sites 5 and 7 throughout the study appeared black, viscous and oily and smelled strongly of oil. Site 6 is on a different bay of Holiday Lake than site 5 and the flow of water is from site 6 toward site 7. No oil slick was observed at site 6 or at site 9 which is on a separate arm of the Lake and did not appear to receive oil as a result of the major spill in 1970. The water level of the Lake is not lowered during the winter. Two sites on Lake Erie (Fig. 3) were chosen for their proximity

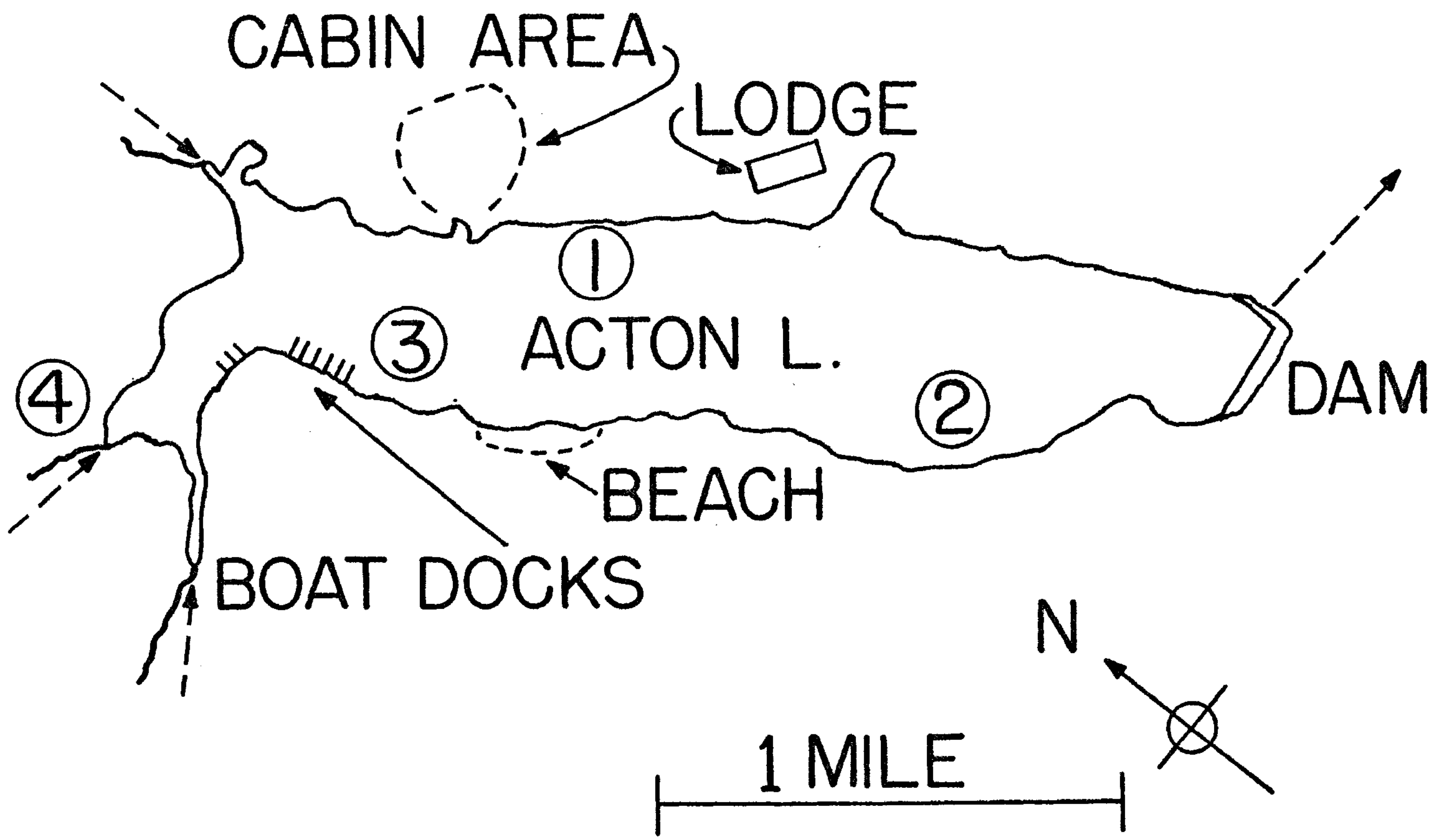

Fig. 1. Acton Lake and sampling sites. 1-4 indicate sampling sites. Dashed arrows indicate the direction of water movement into or out of the Lake.

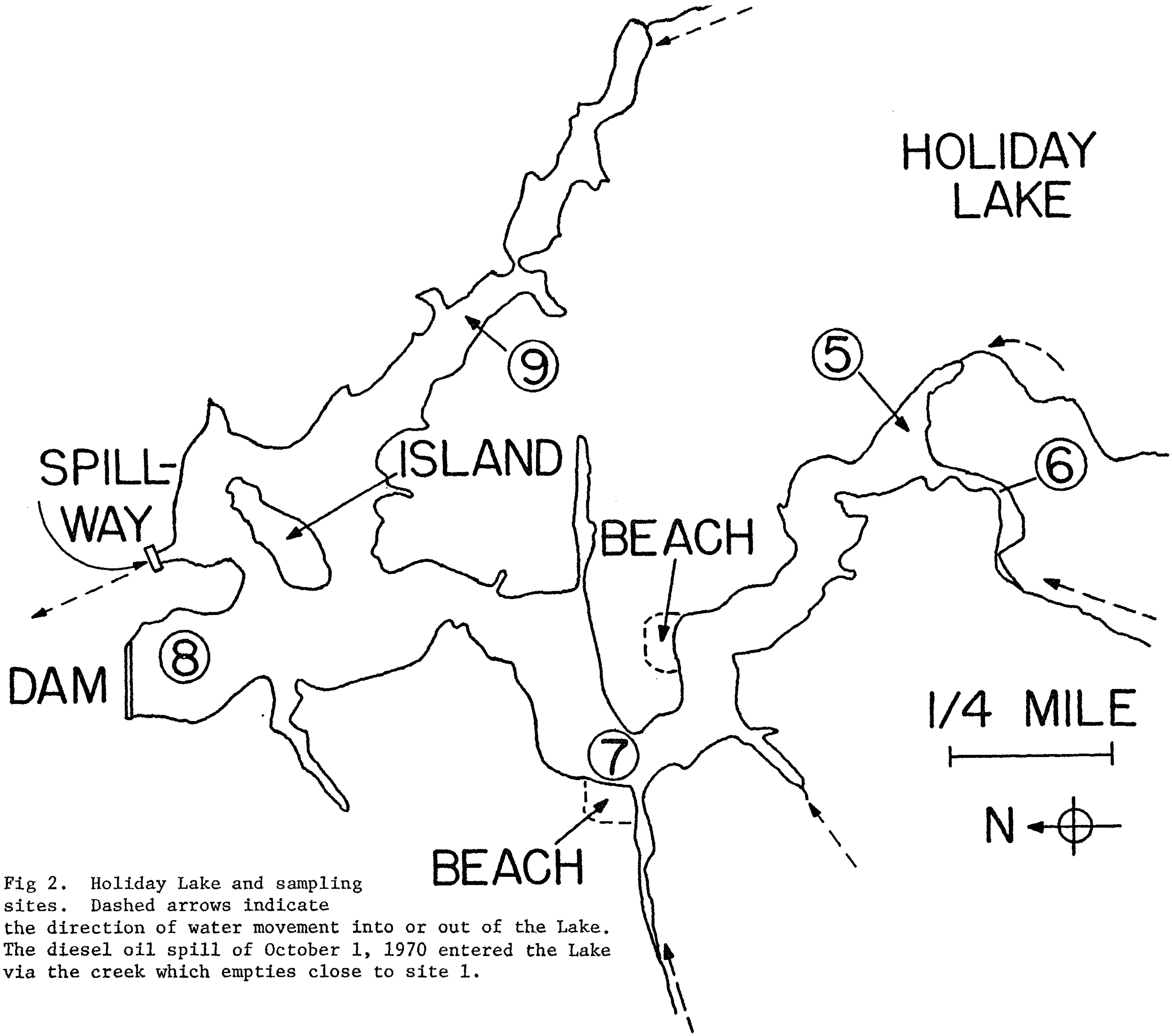

Fig 2. Holiday Lake and sampling sites. Dashed arrows indicate the direction of water movement into or out of the Lake. The diesel oil spill of October 1, 1970 entered the Lake via the creek which empties close to site 1.

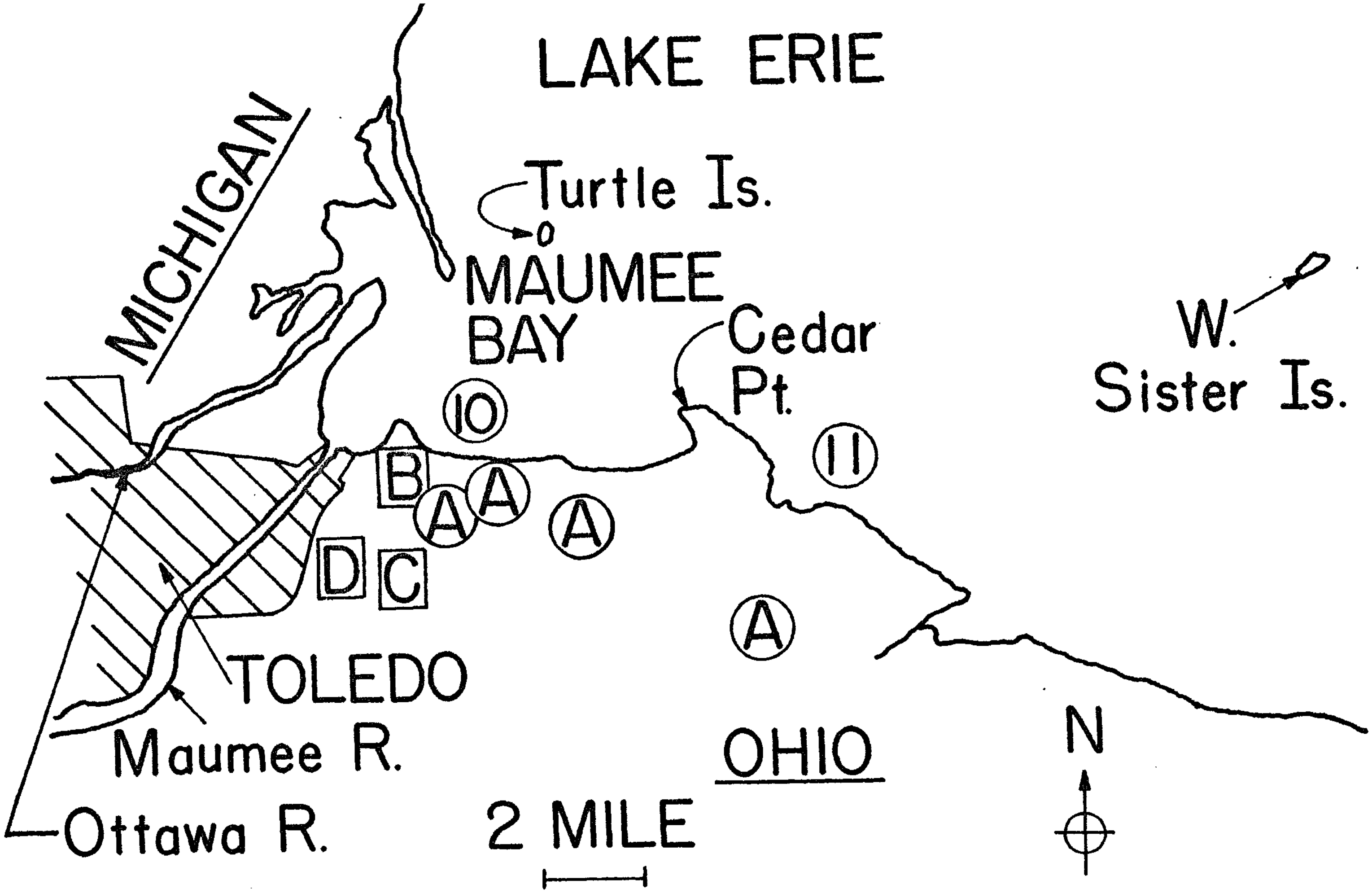

Fig. 3. Lake Erie near Toledo, Ohio. 10 and 11 indicate sampling sites; A designates radio and television masts used for orientation; B, C and D indicate oil storage, pipeline terminal and refinery facilities. respectively

to oil refineries and storage areas. Site 10 is 1/2 mile offshore and close to a pipeline terminal and to oil refinery, oil storage, and coal-powered electric generating facilities. The water flow is eastward from site 10. Site 11 is approximately 5 miles east of site 10 and is also 1/2 mile from shore. Sites on the Miami River (Fig. 4) were selected in an attempt to evaluate the effect of a major industrial area on hydrocarbon-pollution. Samples were taken at mile 99 at the Elizabeth Road Bridge in Tipp City (Site 12) and at mile 66 at the Linden Avenue Bridge in Miamisburg (Site 13). Samples at sites 12 and 13 were taken in the middle of the watercourse. Site 14 was close to shore near site 13. The water is shallow at site 14 and the current is slower than at site 13. At each site, the Miami Valley Conservancy District monitors a number of water parameters which are available as supplementary data for the present work.

Each site was sampled monthly during April through September and bimonthly during October through March. At each site, the bottom was sampled using an Eckman Dredge and the water column was sampled using a Kemmerer Sampler. Where the depth was greater than 8 ft, two water samples were taken, a surface sample and a sample at the midpoint of the water column.

Microbiological methods. The temperature of each sample was determined on site, the sample was iced and returned to the laboratory. On several occasions duplicate microbiological counts were made - in the field and in the laboratory - to establish that counts did not change during transport to the laboratory.

The population of total viable bacteria was estimated by plating appropriate dilutions on the surface of Plate Count Agar (Difco) supplemented with 0.1% (vol/vol) amphotericin B (E.R. Squibb, Inc.) to inhibit fungi. The population of yeast and filamentous fungi was estimated by plating on the surface of Littman Oxgall Agar containing crystal violet (Difco). The medium was supplemented with 30mg streptomycin sulfate per liter to inhibit bacteria. Plates for total viable bacteria or total viable yeast and fungi were incubated for 48h at room temperature prior to counting them.

Hydrocarbon-using populations were estimated by the most probable numbers (MPN) technique as described by Cochran (1949), which proved to be better than plate counts on any of several solid media which were tested. Media for estimation of hydrocarbon-using microorganisms were prepared using the salts solution of Bushnell and Haas (1941) (BHS) which contained: $MgSO_4 \cdot 7H_2O$, 0.2g;

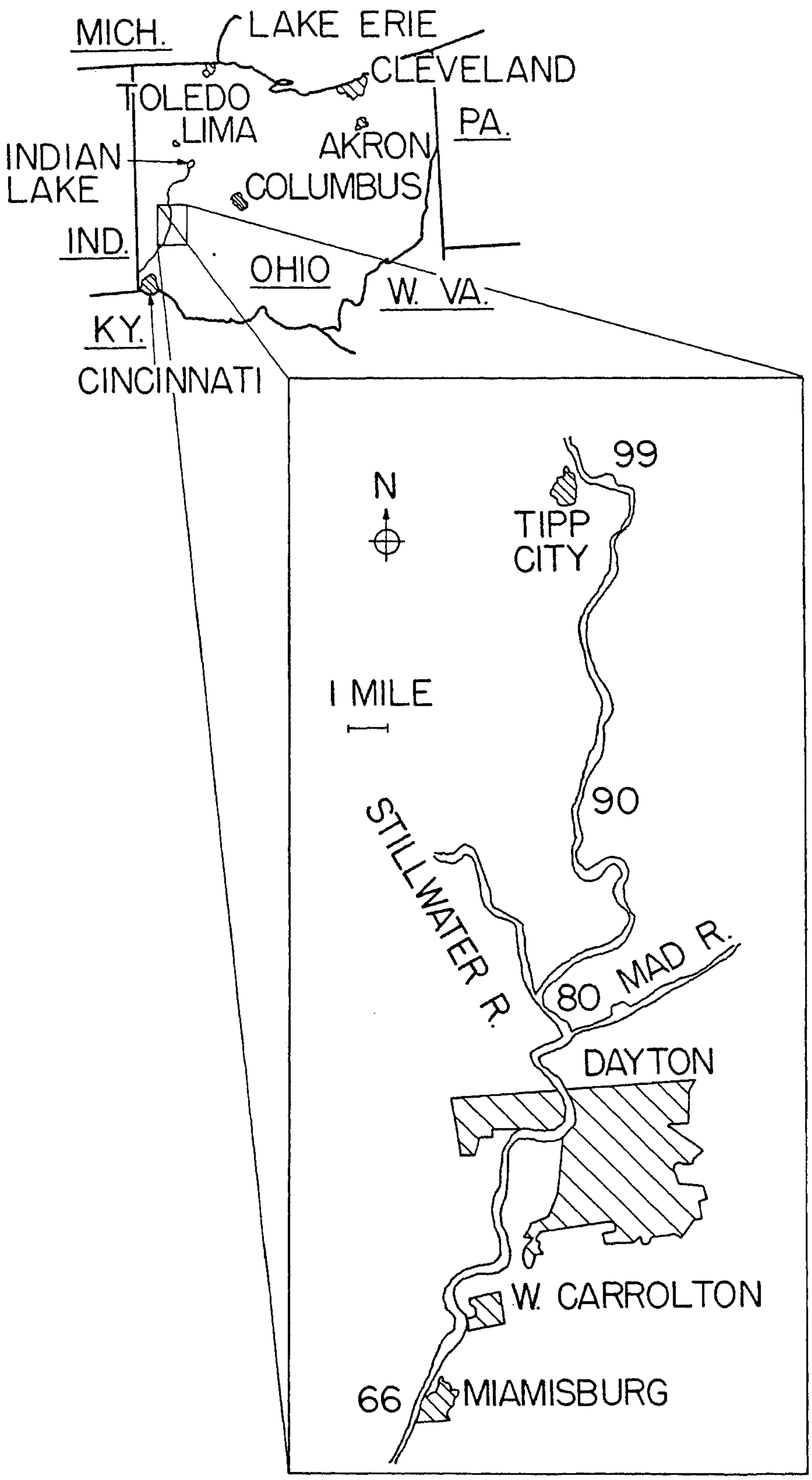

Fig. 4. The Miami River and sampling sites. Numbers indicate mileage upriver from the confluence of the Miami and Ohio rivers. Samples were taken at mile 99 (Site 12) and at mile 66 (Sites 13 and 14).

$CaCl_2$, 0.02g; KH_2PO_4, 1.0g; K_2HPO_4, 1.0g; NH_4NO_3, 1.0g; $FeCl_3$, 2 drops of a solution containing 15g per 25ml H_2O and deionized water, 1000ml. For bacteria the pH of the salts solution was adjusted to 7.0 and the solution was supplemented with 0.1% (vol/vol) amphotericin B. For yeasts and filamentous fungi the pH was adjusted to 5.5 and the solution was supplemented with 1.43mg crystal violet and 30mg streptomycin sulfate per liter. Test tubes, each containing 10ml of sterile BHS, were overlaid with six drops of a 1:1 (vol/vol) mixture of filter-sterilized kerosene and non-detergent SAE 10W motor oil which served as carbon source. After inoculation, tubes were incubated at room temperature for 14 days. A tube was scored as positive for growth if it became turbid, if it contained a pellicle at the oil-water interface or if there was evidence of an emulsion when the tube was compared to a sterile control.

For each sample, several tubes which showed growth were selected and a loopful was streaked on Tryptic Soy Agar (TSA, Difco) or Sabouraud Dextrose Agar (SDA, Difco) to isolate bacteria, and yeasts and filamentous fungi, respectively. Representative colonies were picked and streaked on slants of TSA or SDA and the cultures were added to the collection of hydrocarbon-using organisms.

The gram reaction and cellular morphology of each bacterium was recorded. Fungi were separated into yeasts and filamentous fungi on the basis of colonial morphology and microscopic examination.

Each isolate in the collection was cultured in BHS with kerosene as carbon source. Cultures were scored for rate of growth, for amount of growth and for ability to emulsify the mixture of hydrocarbons. Fifty organisms have been selected for rapid growth and/or ability to emulsify hydrocarbons. Taxonomic studies were conducted on a number of the bacteria in the group of fifty organisms, using standard criteria including cellular and colonial morphology, motility, staining reactions, and physiological and biochemical reactions.

Chemical methods. Each sample was extracted to determine how much carbon it contained. In early experiments, chloroform was used, but samples which contained plant debris also yielded tannins and lignins to chloroform. Therefore, samples were extracted with methylene chloride. For sediment samples, 250ml of a thick slurry were diluted to 1 liter with distilled water prior to extraction. For water samples, 1-liter quantities were extracted. The sample was extracted with three 50-ml quantities of methylene chloride. The combined methylene chloride extracts were filtered through Whatman No. 2 filter paper to remove any suspended solids. The filtered extract was allowed to stand in a separatory funnel until the water phase separated. The methylene chloride layer was separated and dried over anhydrous sodium sulfate for 8 to 12h and then filtered through Whatman No. 2 paper to remove the solid. The filtered extract was reduced to a volume of about 10ml in a rotary evaporator and it was then transferred quantitatively to a pre-weighed aluminum cup. The solvent was allowed to evaporate to dryness overnight and the amount of hydrocarbon was determined after weighing the cup. Hydrocarbons were fractionated into paraffins, olefins and aromatics by column chromatography on silica gel (ASTM, 1967).

RESULTS AND DISCUSSION

1. Comparison of the four ecosystems.

a) Hydrocarbons. For the first year of the study, water and sediment camples were taken at every sampling site in the four ecosystems. Regardless of their source, water samples did not differ significantly in hydrocarbon content or in microbial populations. Thus, in these freshwater ecosystems hydrocarbons which accumulate do so in the sediments and attention should be directed at the sediments and at the sediment-water interface. During the second year of the study, only sediment samples were taken. In contrast to these results Walker and Colwell (1973) detected differences in the hydrocarbon content of water samples between polluted and non-polluted areas of Chesapeake Bay, although sediments contained much more hydrocarbon than water samples.

Sediments may enhance microbial activity by contributing nutrients and by serving as surfaces for attachment of microorganisms. The effect of particulate matter on microbial activity is not clear but it depends in part on the nature of the particles. Weaver and Dugan (1972) found that several clay materials enhanced bacterial methane oxidation while some non-clay inorganic particulates inhibited it. Insoluble organic materials were degraded and did not function as particulates. Wirsen and Jannasch (1974) reported that microbial transformations of various substrates in seawater were stimulated initially by addition of bottom sediments, but the stimulation was followed by a slight depression of activity.

When the data for hydrocarbon content of sediments are grouped (all sites combined) by ecosystem (Table 1) it is evident that bottom samples from the Miami River yielded the largest amount of hydrocarbon and bottom samples from Lake Erie yielded the least hydrocarbon. The large variation around the mean for each ecosystem reflects the variation inherent in grab samples of the bottom. It may also reflect the tendency of persistent hydrocarbons to aggregate into "tarballs" which sink to the bottom (Davis, 1967; Horn et al., 1970). For the Miami River high values and large variation may be due to greater fluctuation in flow rate in the River than in the three lakes. In addition, the samples taken at site 12 on the River were taken from a bridge which has

Table 1. Comparison of the amount of hydrocarbons in sediments from four ecosystems

Ecosystem	Number of sites	Number of samples	mg Hydrocarbon per liter of sediment (Mean $\pm$ SD)	Probability of significant difference between ecosystem and: Acton Lake	Holiday Lake	Lake Erie	Miami River
Acton Lake	4	27	17.46 $\pm$ 20.30		N.S.[1]	$>.95$	$>.95$
Holiday Lake	5	53	30.70 $\pm$ 36.78	N.S.		$>.95$	$>.95$
Lake Erie	2	14	5.39 $\pm$ 6.36	$>.95$	$>.95$		N.S.
Miami River	3	26	72.60 $\pm$ 155.87	$>.95$	$>.95$	N.S.	

[1] The notation N.S. indicates not significant at the 95% confidence interval.

an asphalt surface. Bottom samples from site 12 varied markedly and on two occasions the sample contained a chunk of asphalt, suggesting that some hydrocarbons were derived from bridge material. Although the values for the Miami River are included in all analyses, the large variance decreases the confidence in any conclusions drawn from these data. Thus, the variance of values for the Miami River resulted in a lack of statistical significance between the River and Lake Erie (Table 1) even though the means differ greatly. It is concluded that sampling techniques were inadequate to yield valid data for site 12 in the River.

When the grouped data were treated by analysis of variance, the results support the conclusion that sediments from Lake Erie contained less hydrocarbon than sediments from Holiday Lake or Acton Lake (Table 1). When values for Acton Lake sediments were grouped and compared with grouped values for Holiday Lake sediments, the differences are not significant at the 95% confidence interval (Table 1), in spite of the fact that Acton Lake has a low imput of hydrocarbons and Holiday Lake has been the subject of both acute and chronic hydrocarbon pollution. However, comparison of the four ecosystems requires grouping the data from all the sites in a body of water which masks the significance of hydrocarbons at a particular site. For example, when the hydrocarbon in sediments from individual sites in Holiday Lake is compared with hydrocarbon in Acton Lake sediments, it is evident that site 5 contained significantly more hydrocarbon than did Acton Lake (Table 2).

Kerosene and light motor oil were used as examples of the kinds of hydrocarbons introduced into these systems by man. Paraffins constituted the major class of compounds in both cases (Table 3). Polak and Lu (1973) determined that aromatics are less soluble in water at low temperatures but paraffins are more soluble at low temperatures. Kerosene and motor oil (1:1, vol:vol) were used as carbon source for isolation of hydrocarbon-using organisms in the MPN method. In one experiment the residual oil in the culture vessel was analyzed after 14 days. Olefins persisted and aromatics and paraffins had decreased. Therefore, the ecosystems contain microorganisms which are capable of growing on aromatics and paraffins. Olefins constituted the major fraction of hydrocarbons extracted from sediments. Thus, paraffins comprise the largest class of hydrocarbons introduced into any of the four ecosystems. But they do not persist due to a combination of weathering (Nelson-Smith, 1970) and microbial utilization. Olefins persist and should be the subject of future attempts to deal with the effects of long term hydrocarbon pollution.

Table 2. Comparison of the amount of hydrocarbon in sediments from Acton Lake and sediments from individual Holiday Lake sites

Site in Holiday Lake	Probability of significant difference between site and Acton Lake
5	$>.99$
6	N.S.[1]
7	N.S.
8	N.S.
9	N.S.

1 The notation N.S. indicates not significant at the 95% confidence interval.

Table 3. Composition of hydrocarbons

Hydrocarbons from:	Number of sites	Number of samples	Volume per cent		
			Aromatics (Mean ± SD)	Olefins (Mean ± SD)	Paraffins (Mean ± SD)
Kerosene		2	15.0 ± 5.0	28.8 ± 1.0	56.1 ± 4.2
Motor oil		2	10.5 ± 0.7	27.5 ± 1.1	61.6 ± 1.3
Hydrocarbon medium after 14 days growth		1	8.5	82.3	9.2
Acton Lake	4	24	14.8 ± 5.8	64.9 ± 12.1	20.3 ± 12.7
Holiday Lake	5	41	19.4 ± 10.6	61.3 ± 16.1	18.8 ± 11.5
Lake Erie	2	10	24.0 ± 23.0	58.7 ± 25.4	16.9 ± 16.6
Miami River	3	19	22.9 ± 8.5	53.7 ± 16.8	23.1 ± 12.4

b) Microbial populations. The grouped values for total viable bacterial populations of the four ecosystems are compared in Table 4. Sediments from Lake Erie had a lower bacterial population than the other systems examined, and the differences are significant at the 99% level. Sediments from Acton Lake contained significantly fewer viable bacteria than sediments from Holiday Lake, but populations in Holiday Lake or Acton Lake are not significantly different from the populations in the Miami River.

Table 5 compares the grouped data for total numbers of yeasts and fungi in sediments from the four ecosystems. In each ecosystem, averages were two logarithms lower than for total viable bacteria (Table 4). Individual cells of yeasts and filamentous fungi, however, are larger than bacterial cells. Furthermore, filamentous fungi exist in large masses of cells. Therefore, one colony-forming unit for yeasts or fungi represents a much larger biomass than a colony-forming unit for bacteria, and the relative biomasses are undoubtedly much closer than the hundred-fold difference indicated. The numbers of yeasts and fungi are significantly lower in sediments from Lake Erie than in sediments from the other bodies of water, but other differences were not significant (Table 5).

Grouped data for hydrocarbon-using bacteria in sediments from the four ecosystems is provided in Table 6. In each case, hydrocarbon-users represented only 0.1% of the total bacterial population (Table 4). Sediments from Lake Erie yielded the least hydrocarbon-using bacteria and sediments from the Miami River yielded the greatest number. Counts for Holiday Lake and the Miami River were each significantly different from counts for Lake Erie and Acton Lake (Table 6). Other differences were not statistically significant. Walker and Colwell (1973) reported numbers of petroleum users similar to those reported here in sediments from Chesapeake Bay. They stated that 26 to 75 % of the total viable bacteria used hydrocarbon when tested on a solid medium containing Difco purified agar. In our preliminary experiments, however, we noted that Difco purified agar supported growth of many organisms which did not grow on hydrocarbons. Thus, the relatively high percentages of hydrocarbon-users reported for Chesapeake Bay may include some organisms growing on impurities in the agar.

Grouped data for hydrocarbon-using yeasts and filamentous fungi are compared in Table 7. Lake Erie sediments yielded the smallest number (less than 1 propagule per 10 ml) and Holiday Lake yielded the largest number.

Table 4. Comparison of the total viable bacterial population in sediments from four ecosystems

Ecosystem	Number of sites	Number of samples	Log of bacterial count/ml sediment (Mean $\pm$ SD)	Probability of significant difference between ecosystem and: Acton Lake	Holiday Lake	Lake Erie	Miami River
Acton Lake	4	39	6.5 ± 1.2		$>.95$	$>.99$	N.S.[1]
Holiday Lake	5	61	7.1 ± 1.1	$>.95$		$>.99$	N.S.
Lake Erie	2	18	5.3 ± 1.3	$>.99$	$>.99$		$>.99$
Miami River	3	31	6.8 ± 1.3	N.S.	N.S.	$>.99$	

[1] The notation N.S. indicates not significant at the 95% confidence interval.

Table 5. Comparison of the total viable yeast and filamentous fungal population in sediments from four ecosystems

				Probability of significant difference between ecosystem and:			
Ecosystem	Number of sites	Number of samples	Log of yeast and fungal count/ml sediment (Mean $\pm$ SD)	Acton Lake	Holiday Lake	Lake Erie	Miami River
Acton Lake	4	39	4.8 ± 1.2		N.S.[1]	>.99	N.S.
Holiday Lake	5	60	4.4 ± 1.2	N.S.		>.99	N.S.
Lake Erie	2	18	3.0 ± 1.4	>.99	>.99		>.99
Miami River	3	31	4.5 ± 1.0	N.S.	N.S.	>.99	

[1] The notation N.S. indicates not significant at the 95% confidence interval.

Table 6. Comparison of the hydrocarbon-utilizing bacterial population in sediments from four ecosystems

Ecosystem	Number of sites	Number of samples	Log of bacterial count/ml sediment (Mean ± SD)	Probability of significant difference between ecosystem and: Acton Lake	Holiday Lake	Lake Erie	Miami River
Acton Lake	4	23	3.0 ± 0.6		>.95	N.S.[1]	>.99
Holiday Lake	5	43	3.6 ± 1.1	>.95		>.99	N.S.
Lake Erie	2	12	2.0 ± 2.4	N.S.	>.99		>.99
Miami River	3	23	3.7 ± 0.8	>.99	N.S.	>.99	

1 The notation N.S. indicates not significant at the 95% confidence interval.

Table 7. Comparison of the hydrocarbon-utilizing yeast and fungal population in sediments from four ecosystems

Ecosystem	Number of sites	Number of samples	Log of yeast and fungal count/ml sediment (Mean $\pm$ SD)	Probability of significant difference between ecosystem and:			
				Acton Lake	Holiday Lake	Lake Erie	Miami River
Acton Lake	4	24	0.6 ± 1.2		$>.95$	$>.99$	N.S.[1]
Holiday Lake	5	43	1.1 ± 0.8	$>.95$		$>.99$	$>.99$
Lake Erie	2	12	-1.2 ± 0.9	$>.99$	$>.99$		N.S.
Miami River	3	23	0.2 ± 1.8	N.S.	$>.99$	N.S.	

[1] The notation N.S. indicates not significant at the 95% confidence interval.

In each case hydrocarbon-using fungi comprised a small fraction of the total fungal population (Table 5). Counts were lower than for hydrocarbon-using bacteria (Table 6), but the size of individual cells and of colony-forming units are larger than for bacteria, indicating that the relative biomasses of the two groups are much closer. Even when grouped by ecosystem, Holiday Lake yielded significantly larger numbers of hydrocarbon-using fungi than any of the other ecosystems (Table 7). Values for Acton Lake are significantly different from values for Lake Erie.

c) Summary. Differences beyween ecosystems were found in the sediments rather than in the water column. Since grab samples yield not only sediments but water near the bottom, the sediment or bottom samples are actually samples of the bottom materials plus water. Continuing studies are in progress on the sediments and the sediment-water interface as the potential site of action on persistent hydrocarbons.

When the four ecosystems are compared as ecosystems, i.e. when all the data for sediments from a single ecosystem are grouped, the grouping tends to decrease differences between the ecosystems. For example, values for the marina at Acton Lake (Site 3) increase the mean values for the entire Lake and values for sites 6 and 9 at Holiday Lake decrease the mean values for the entire Lake. In spite of this tendency to smooth the data, Lake Erie contained significantly less hydrocarbon than either of the recreational lakes, and a specific site in Holiday Lake differed significantly from grouped data for Acton Lake. Although paraffins constitute the major class of hydrocarbons likely to enter each ecosystem, olefins are the major class of persistent compounds and future efforts should be directed at olefins.

Based on these analyses of grouped data, little correlation is evident between hydrocarbon content of sediments and total microbial populations, suggesting that oil pollution does not inhibit or enhance the numbers of heterotrophic microorganisms. However, a trend is suggested wherein ecosystems which contain more hydrocarbons also yield larger numbers of hydrocarbon-using microorganisms even though the hydrocarbon-users constitute only a small fraction of the total microbial population. In all cases - total populations or hydrocarbon-users - the numbers of bacteria far exceed the numbers of yeasts and filamentous fungi, but the relative biomasses may be more nearly equal.

2. Comparisons within ecosystems.

Data for individual sites in each ecosystem are presented in Table 8 and the results of applying analysis of variance techniques within each ecosystem are presented in Table 9.

a) Hydrocarbons. Sediments from the marina (Site 3) at Acton Lake yielded the greatest amounts of hydrocarbon and sediments from the inlet stream (Site 4) yielded the least hydrocarbon. Analysis of variance indicated that all values combined could have been drawn from the same population (Table 9). However, sediments at the marina yielded significantly more hydrocarbon than a mid-lake site and differences between the marina and the main inlet stream were close to significance ($p=0.91$). These data suggest that the operation of a number of small outboard engines in the relatively confined area of the marina contributes oil to the ecosystem. The hydrocarbons had the same composition of paraffins, olefins and aromatics as hydrocarbons from other sites, indicating that the same general groups of compounds persist whether they are derived from petroleum as at the marina or from plant and animal material as in the stream.

In Holiday Lake, the variance in data for all sites combined indicates that samples were not drawn from the same population. Sediments at site 5 yielded significantly more hydrocarbon than any other site in the Lake. Site 6 was close to significance ($p=0.94$), but sediments did not appear oily, nor did they have an oily odor. In contrast, site 7 is not different from site 8 ($p=0.91$). It is clear that considerable hydrocarbons entered Holiday Lake through the stream which enters at site 5, and it appears that additional hydrocarbons entered during the course of this investigation. During the two years of this study the water depth at site 5 decreased from 2 ft to about 6 in. due to silting in. But as silting progressed the hydrocarbon content of the sediment-water samples did not decrease, leading to the conclusion that hydrocarbon pollution of Holiday Lake is continuous. Potential sources are a sewage treatment plant and a railroad repair and maintenance shop which contribute water to the stream. Effluent water from the railroad shop enters a holding pond. The shores of the pond are saturated with oil and on a number of occasions the surface of the pond was covered with oil. A mechanical skimmer is intended to remove oil before water leaves the pond and a weir is located downstream from the pond. The weir is intended to retain any oil which might escape from the pond. During the two years of this study, no oil was observed on the surface behind the weir. However, water leaving the holding pond probably contains near-saturation levels of oil. The sewage

Table 8. Amount of hydrocarbons and the microbial populations of sites within each ecosystem

Sites	mg Hydrocarbons per liter of sediment (Mean ± SD)	Log of Total viable bacteria/ml (Mean ± SD)	Log of Total viable yeast & fungi/ml (Mean ± SD)	Log of Hydrocarbon-utilizing bacteria/ml (Mean ± SD)	Log of Hydrocarbon-utilizing yeast & fungi/ml (Mean ± SD)
Acton Lake:					
1.	15.4 ± 18.7	6.5 ± 1.2	4.6 ± 1.1	3.1 ± 0.9	0.7 ± 1.2
2.	19.9 ± 26.9	6.7 ± 1.0	4.0 ± 1.1	2.6 ± 0.4	0.6 ± 1.1
3.	31.2 ± 22.0	6.6 ± 1.3	4.2 ± 1.3	3.4 ± 0.8	0.6 ± 1.8
4.	11.7 ± 16.6	6.6 ± 1.3	4.6 ± 1.0	2.9 ± 0.2	-0.4 ± 1.2
Holiday Lake:					
5.	69.9 ± 53.0	7.2 ± 1.4	5.5 ± 1.3	4.5 ± 0.7	1.7 ± 0.8
6.	35.5 ± 38.9	7.1 ± 1.0	4.6 ± 1.0	3.6 ± 0.9	1.0 ± 0.6
7.	27.8 ± 30.0	7.2 ± 0.9	3.8 ± 1.1	3.6 ± 1.2	1.1 ± 0.6
8.	10.2 ± 7.9	6.8 ± 1.1	3.6 ± 0.7	3.2 ± 0.9	0.7 ± 1.0
9.	16.2 ± 15.9	6.7 ± 0.9	4.3 ± 1.0	3.3 ± 1.2	0.7 ± 0.4
Lake Erie:					
10.	6.4 ± 7.2	5.8 ± 0.9	3.7 ± 1.0	3.3 ± 1.6	-1.2 ± 1.2
11.	4.4 ± 5.7	4.8 ± 1.4	2.4 ± 1.6	0.7 ± 2.6	-1.2 ± 0.9
Miami River:					
12.	160.4 ± 260.0	6.2 ± 1.3	4.2 ± 0.9	3.4 ± 0.7	-1.2 ± 1.0
13.	30.9 ± 33.7	7.3 ± 1.1	4.5 ± 1.1	3.5 ± 0.9	-0.5 ± 1.7
14.	38.6 ± 73.6	7.3 ± 1.1	4.6 ± 0.8	4.3 ± 0.4	0.4 ± 1.9

Table 9. Probability of significant difference in the amount of hydrocarbons and the microbial populations in sediments between sites within each ecosystem

	Probability of significant difference				
Site vs site	Hydrocarbons	Total viable bacteria	Total viable yeast & fungi	Hydrocarbon-utilizing bacteria	Hydrocarbon-utilizing yeast & fungi
Acton Lake					
1 vs 2	N.S.[1]	N.S.	N.S.	N.S.	N.S.
1 vs 3	>.98	N.S.	N.S.	N.S.	N.S.
1 vs 4	N.S.	N.S.	N.S.	N.S.	>.98
2 vs 3	N.S.	N.S.	N.S.	N.S.	N.S.
2 vs 4	N.S.	N.S.	N.S.	N.S.	>.95
3 vs 4	>.91	N.S.	N.S.	N.S.	N.S.
All sites combined	N.S.	N.S.	N.S.	N.S.	N.S.
Holiday Lake					
5 vs 6	>.96	N.S.	>.94	>.97	>.93
5 vs 7	>.99	N.S.	>.99	>.92	N.S.
5 vs 8	>.99	N.S.	>.99	>.99	>.95
5 vs 9	>.99	>.92	>.98	>.98	>.98
6 vs 7	N.S.	N.S.	>.94	N.S.	N.S.
6 vs 8	>.94	N.S.	>.98	N.S.	N.S.
6 vs 9	N.S.	N.S.	N.S.	N.S.	N.S.
7 vs 8	>.91	N.S.	N.S.	N.S.	N.S.
7 vs 9	N.S.	N.S.	N.S.	N.S.	>.98
8 vs 9	N.S.	N.S.	>.91	N.S.	N.S.
All sites combined	>.99	N.S.	>.99	>.90	>.96
Lake Erie					
10 vs 11	N.S.	>.93	>.95	>.94	N.S.
Miami River					
12 vs 13	N.S.	>.95	N.S.	N.S.	>.95
12 vs 14	N.S.	>.93	N.S.	>.98	>.93
13 vs 14	N.S.	N.S.	N.S.	>.91	N.S.
All sites combined	N.S.	>.93	N.S.	>.93	>.93

1 The notation N.S. indicates not significant at the 90% confidence interval.

treatment plant had at least one breakdown of a trickling filter during the study. Moreover, after rainstorms a large fraction of the water entering the plant receives minimal treatment. On several occasions it was noted that the effluent from the sewage treatment plant made the water turbid in the stream before it entered the Lake. Therefore, either or both of these potential sources could be contributing hydrocarbons to Holiday Lake. Two other sources of hydrocarbons are the motors of pleasure boats and run-off from farms. It is estimated that 1.0-1.6 x 10^6 gallons of fuel per year are discharged into American waters by two-cycle outboard engines. At concentrations of 5 ppm or less, raw fuel or crankcase drainage from outboard engines inhibits CO_2 fixation by algae. (R.P.I., 1972). But boating should not have contributed to the difference between site 5 and other sites because the shallow depth at site 5 decreased boat traffic and the water flow is away from site 5 into the main body of the Lake. Agricultural run-off is considered to be the major source of hydrocarbons at site 6, but not at site 5 because of the visible differences in sediment samples. On two occasions during the study surface blooms of algae were observed in the areas of sites 5 and 6.

The amount of hydrocarbons in sediments from Lake Erie were not greater close to oil and coal facilities (Site 10) than at a point 5 miles away (Site 11).

Samples at site 12 in the Miami River showed great variation (Table 8), which rendered the differences insignificant at the 90% confidence level. As indicated above, the variation may be due to chunks of asphalt which had fallen into the River at site 12. Samples taken at sites 13 and 14 were not contaminated with bridge materials. Neither the hydrocarbon content of the two sites nor the microbial populations were significantly different between the midstream site and the site near shore.

b) Microbial populations. For Acton Lake the differences in microbial populations were not significant, except that sediments from the stream (Site 4) yielded significantly fewer hydrocarbon using yeasts and fungi (less than one per ml) than sediments from sites 1 and 2 in the Lake (Tables 8 and 9).

Sediments from Holiday Lake did not differ significantly in their total viable bacterial population regardless of the site. For total viable yeasts and fungi analysis of all sites combined (Table 9) suggested that more than one population was sampled. Sites 5 and 6 yielded significantly larger numbers of yeasts and filamentous fungi than other sites. Site 5 contained more hydrocarbon-using organisms of both types than other sites, and site 7 yielded significantly more hydrocarbon-using yeasts and fungi than site 9. Therefore, sediments from Holiday Lake which contained large amounts of hydrocarbon contain larger populations of yeasts and fungi, of hydrocarbon-using bacteria and of hydrocarbon-using yeasts and filamentous fungi. Walker and Colwell (1973) reported that the numbers of petroleum-degrading bacteria in sediments from Chesapeake Bay were related to the concentration of oil in each sample.

Sediments from site 10 in Lake Erie contained more yeasts and filamentous fungi than site 11, but other differences were not significant at the 95% confidence interval. Both sites at Lake Erie and both midstream sites in the Miami River yielded averages of less than one propagule of hydrocarbon-using fungi per 10 ml (Table 8). Although some significant differences among populations were apparent at the river sites, (Table 9) it is difficult to interpret them because of variation in samples at site 12.

c) Linear regression analysis. In an attempt to determine if the sizes of the microbial populations in an ecosystem correlated with some key parameters, the data for Acton and Holiday Lakes and the Miami River were analyzed using a step-wise multiple regression technique. Data for the Miami River were included because the analysis is designed to deal with variations from sample to sample. Data for Lake Erie were not analyzed by this technique because only two sites were involved. For each of the four microbial populations the following variables were entered in the regression analysis: total hydrocarbon content of the sediment, individual amounts of aromatic, olefinic and paraffinic hydrocarbons, individual percentages of aromatic, olefinic and paraffinic hydrocarbons, each of the other three microbial populations and each individual sampling site in the ecosystem.

Significant correlations for Acton Lake, Holiday Lake and the Miami River are summarized in Tables 10, 11 and 12, respectively. In all three ecosystems, the numbers of total viable bacteria showed a strong positive correlation with the total number of yeasts and filamentous fungi. Thus, the total population of heterotrophic microorganisms appears to vary together. In all three ecosystems, there were some negative correlations between amount or per cent of aromatics and olefins suggesting that these fractions of petroleum products may be inhibitory or toxic to the microbial flora. However, these correlations were not consistent enough to support a firm conclusion. In Acton Lake the total bacterial population correlated positively with the amount of paraffins and the per cent of olefins and negatively with the amount of aromatics in the sediments. The total fungal population correlated with the population of

Table 10. A summary of the variables correlating with the microbial populations from Acton Lake determined using a step-wise multiple regression technique.

Microbial population	Variable (in order of selection)	Type of correlation	Correlation coefficient for this group of variables	Probability of true correlation
Total viable bacteria	Total viable yeast & fungi	+	.69	>.99
	Amount of paraffins	+		>.99
	Per cent of olefins	+		>.99
	Amount of aromatics	-		>.98
Total viable yeast & fungi	Hydrocarbon-utilizing bacteria	+	.68	>.99
Hydrocarbon-utilizing bacteria	Total viable yeast & fungi	+	.68	>.99
Hydrocarbon-utilizing yeast & fungi	Site 4	-	.45	>.97

Table 11. A summary of the variables correlating with the microbial populations from Holiday Lake determined using a step-wise multiple regression technique.

Microbial population	Variable (in order of selection)	Type of correlation	Correlation coefficient for this group of variables	Probability of true correlation
Total viable bacteria	Total viable yeast & fungi	+	.78	1
	Site 7	+		>.99
	Hydrocarbon-utilizing bacteria	+		>.98
Total viable yeast & fungi	Hydrocarbon-utilizing yeast & fungi	+	.62	>.99
	Total viable bacteria	+		>.96
Hydrocarbon-utilizing bacteria	Hydrocarbon-utilizing yeast & fungi	+	.76	1
	Per cent paraffins	-		>.99
Hydrocarbon-utilizing yeast & fungi	Hydrocarbon-utilizing bacteria	+	.66	1
	Per cent olefins	-		>.97

Table 12. A summary of the variables correlating with the microbial populations from the Miami River determined using a step-wise multiple regression technique.

Microbial population	Variable (in order of selection)	Type of correlation	Correlation coefficient for this group of variables	Probability of true correlation
Total viable bacteria	Total viable yeast & fungi	+	.84	1
	Per cent of olefins	-		>.97
Total viable yeast & fungi	Total viable bacteria	+	.88	>.99
	Hydrocarbon-utilizing yeast & fungi	+		>.99
	Per cent of aromatics	-		>.98
Hydrocarbon-utilizing bacteria	Hydrocarbon-utilizing yeast & fungi	+	.60	>.99
	Amount of aromatics	-		>.95
Hydrocarbon-utilizing yeast & fungi	Total viable yeast & fungi	+	.75	>.99
	Site 14	+		>.99

hydrocarbon-using bacteria, but the reason is not evident. As revealed by analysis of variance discussed above, hydrocarbon-using yeasts and fungi showed a negative correlation with the inlet stream.

In Holiday Lake (Table 11) and the Miami River (Table 12), which showed the greatest variation in hydrocarbon contents within the ecosystems, the numbers of hydrocarbon-using bacteria showed a strong positive correlation with the numbers of hydrocarbon-using yeasts and filamentous fungi supporting the suggestion that both groups are important in hydrocarbon-polluted systems.

Bartha and Atlas (1973) reported that hydrocarbon-using bacteria were abundant in seawater, but that the range of substrates attacked by individual organisms was restricted. Thus, degradation of the great variety of hydrocarbons present in crude or refined petroleum probably involves a number of organisms. Beam and Perry (1973) found that cyclohexane was degraded when incubated with fertile soil, but no single organism isolated from the soil could degrade cyclohexane. They suggested that degradation of cycloalkanes in soil proceeds via co-metabolism. Soli and Bens (1973) reported that combined strains of bacteria did not degrade hydrocarbons in flask cultures better than single strains. But Cerniglia and Perry (1973) observed that fungi decompose crude oil better than bacteria. They concluded that no single organism can degrade all fractions of crude oil. Moreover, Fliegler, McNabb and Fields (1974) present data which suggest that fungi inhibit growth of bacteria in aerobic lake sediments. These reports agree with the results of the present study which suggest that bacteria, yeasts and filamentous fungi are all active participants in degradation and mineralization of hydrocarbons in aquatic ecosystems.

3. Kinds of organisms isolated.

Of 353 representative organisms isolated, approximately 55% were bacteria, 33% were yeasts and 13% were filamentous fungi (Table 13). Thus, yeasts and filamentous fungi comprise a major fraction of the microorganisms isolated. The solid media used in early experiments yielded more filamentous fungi than the liquid medium used for the bulk of the study. Therefore, the proportion of filamentous fungi may be larger. Since the yeasts and filamentous fungi have larger cells than bacteria, their relative contribution to the biomass is larger than their numbers suggest. Gram negative rods comprised 77% of the bacteria examined. Of the bacteria which grew on kerosene, 30% were

Table 13. Characterization of microorganisms isolated

Type of organism	Number	Growth noted in kerosene-salts medium after: 1 wk	2 wk	3 wk	4 wk	Not at 4 wk*	Number emulsifying kerosene
Bacteria							
gram positive rods	20	6	5	3	3	3	9
gram positive cocci	6	0	4	1	0	1	1
gram negative rods	127	39	41	29	13	5	30
gram negative cocci	12	0	4	3	0	5	5
unexamined bacteria	28						
Yeasts	114	41	36	18	10	9	33
Filamentous fungi	46	12	14	8	5	7	11
Totals	353	98	104	62	31	30	89

* Includes 10 bacteria and 7 yeasts which were lost upon transfer.

capable of emulsifying the kerosene. Similar percentages of yeasts (31%) and filamentous fungi (28%) produced emulsions. It is possible that additional organisms produced microemulsions which would not have been detected by visual inspection. In each group of organisms the majority of the isolates were capable of growth on kerosene in a period of 2 weeks or less. Of 353 isolates, only 17 (4.8%) were lost when transferred several times on laboratory media.

Ten of the gram negative rods which grew rapidly with kerosene as their carbon source were the subjects of a taxonomic study. Three were identified as strains of *Pseudomonas aeruginosa*, three others as *P. oralis*, *P. dachunae* and *P. striata*, respectively, two as *Cellulomonas acidula* strains, one as a *Flavobacterium* sp. and one as an *Achromobacter* sp. The tenth gram negative rod remains unidentified. It is a non-motile, non-sporeforming organism which forms small, pink colonies on solid medium. It grows in a nutrient gelatin stab without hydrolysing the gelatin. It is catalase-positive and indole-positive, but it does not produce H_2S nor does it reduce nitrate. The organism grows in 0.5% peptone, it does not hydrolyse cellulose and growth is not apparent in litmus milk. It achieves little growth on sugars with weak acid production from sucrose, glucose and lactose.

4. Additional work begun under this grant.

In order to assess the potential of the microorganisms isolated, two studies were begun in the last two months of the grant. They are being continued under a Matching Grant (B-060-OHIO).

Considerable information is available on how pure cultures of microorganisms deal with pure hydrocarbons under laboratory conditions, but relatively little is known of use of hydrocarbons under field conditions. We are attempting to develope a vessel which can be placed on the bottom of a lake or stream or suspended in the water column. Water and light will be able to pass through the vessel, but oil will not pass out. Several suitable filters have been examined which will pass water but not oil. Thus, pure cultures, mixed cultures of known organisms or the natural flora can be added to sediment or water containing a pure hydrocarbon, a mixture of hydrocarbons or a specific petroleum product. The action of the organisms can be examined essentially *in situ* where they will be under the influence of diurnal and seasonal variation.

Selected organisms are being screened for ability to use a variety of hydrocarbons when presented with a mixture of hydrocarbons. A mixture of 22 hydrocarbons has been selected which includes representatives of major classes of compounds present in crude oil and in many classes of refined petroleum (Mair, 1964). The mixture includes cyclopentane, cyclohexane, cyclohexene, ethylbenzene, isopropylbenzene, dodecylbenzene, dodecylcyclohexane, decane, undecane, 2-methylundecane, 1,13-tetradecyldiene, tetradecene-7, dodecane-1, pentadecane, hexadecane, pristane, phytane, phenanthrene, anthracene, naphthalene, eicosane and triacontane. Pure cultures and mixed cultures will be screened for ability to degrade recalcitrant molecules in the presence of a variety of alternate hydrocarbon substates.

5. General Discussion and Conclusions.

The author is not aware of any prior attempts to establish the relative numbers of major microbial groups of hydrocarbon-users in aquatic ecosystems.

The techniques used in this study have several inherent weaknesses. As discussed above, the use of the Eckman dredge or similar grab samplers yields a sample containing material from the zone near the water-sediment interface. The sampling devices available to microbiologists are much less sophisticated than the techniques for analysis of the sample.

The microbial populations detected (counted) in this study are at best an estimate of the total population present in the ecosystems. Cultures were incubated at 28 $\pm$ 2C for 2 days (total populations) or 14 days (hydrocarbon-users). Thus, psychrophilic organisms, which grow at temperatures below 15C, and slow-growing organisms may not have been tabulated. No attempt was made to detect microaerophiles, which grow only in the presence of minute quantities of oxygen, or anaerobes. Each of these types of organisms plus microscopic algae should be measured in order to have a complete picture of the microbial flora. Moreover, the data obtained provide no information as to whether these organisms are active in situ where a variety of substrates is present and where there is competition from and predation by other organisms. Nor has any information been gathered about variations in rates of hydrocarbon metabolism from season to season. Atlas and Bartha (1972b) reported that in seawater biodegradation of oil was slower at 5C than at 20C. In contrast, Wirsen and Jannasch (1974) noted that microbial activity on non-hydrocarbon substrates in seawater was higher in winter than in summer. In many aquatic systems low levels of nitrogen and phosphorous may limit microbial activity severely (Bartha and Atlas, 1973; Cerniglia and Perry, 1973). If so, the activity of microorganisms present in these four ecosystems could possibly be enhanced by judicious seeding with chemicals. A number of these aspects are being dealt with in work summarized above which was begun under this grant, and which is being continued under a Matching Grant. In spite of these limitations, the collection of 350 aerobic, mesophilic hydrocarbon-using microorganisms isolated from these four ecosystems represents a library of representative freshwater organisms on which a number of continuing studies can be based including not only the questions raised above but also such aspects as co-oxidation of recalcitrant substrates (Raymond, Jamison and Hudson, 1971; Horvath, 1972) and cooperative action among microorganisms (de Klerk and van der Linden, 1974).

The following general conclusions and suggestions have been made:

1. While the immediate effects of acute oil pollution are manifest at the water surface and at the shore, long term effects and effects of chronic pollution by petroleum are evident in the bottom of freshwater ecosystems. Future work should be directed to the sediments and the sediment-water interface.

2. Sediments from Lake Erie yielded significantly less hydrocarbon than sediments from two recreational lakes, Holiday Lake and Acton Lake. Sediments from an individual site in Holiday Lake contained significantly more hydrocarbons than sediments from Acton Lake.

3. Aromatic, olefinic and paraffinic hydrocarbons are all present in the persistent fractions of oil in sediments. Although paraffins are the largest clsss of compounds in petroleum when it enters a lake or stream, olefins are the largest class of persistent compounds. Efforts should be directed toward degradation or removal of olefins.

4. The hydrocarbon (oil) content of sediments does not inhibit or enhance the numbers of heterotrophic microorganisms and numbers of bacteria and of yeasts and filamentous fungi varied together. A trend is suggested wherein sediments which contain more hydrocarbons contain more hydrocarbon-using bacteria, yeasts and filamentous fungi.

5. The numbers of bacteria isolated were at least 100 times as great as the numbers of yeasts and filamentous fungi, but the relative biomasses may be more nearly equal.

6. Hydrocarbon-using microorganisms comprised a small fraction (0.1% or less) of the total aerobic microbial population. More hydrocarbon-using bacteria were detected than yeasts and filamentous fungi but the relative biomasses may be more nearly equal.

7. Sediments from a marina at Acton Lake contained significantly more hydrocarbons than sediments from other parts of the Lake.

8. Holiday Lake, which was the site of a major oil spill in 1970, appears to be the subject of continued hydrocarbon input through one of its major inlet streams.

9. Hydrocarbons in sediments from Lake Erie were not greater close to oil and coal facilities than at a site 5 miles away.

10. Linear regression analyses yielded data suggesting that a high content of aromatic and/or olefinic hydrocarbons may be inhibitory or toxic to the microbial flora, but the correlations were not consistent enough to support a firm conclusion.

11. Numbers of hydrocarbon-using bacteria correlated with the numbers of hydrocarbon-using yeasts and filamentous fungi indicating that both groups are important in hydrocarbon-polluted systems.

12. Of 353 representative microorganisms selected during the study approximately 55% were bacteria, 33% were yeasts and 13% were filamentous fungi. In each group of organisms the majority of the isolates grew on kerosene in 2 weeks or less, and approximately 30% of the isolates in each group emulsified kerosene.

13. Gram-negative rods comprised 77% of the bacteria selected. Nine of these were characterized to the generic level or the species level.

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